



Patent

Attorney's Docket No. 1032751-000027

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of	)	
Jean-Marc Balloul et al.	)	Group Art Unit: 1648
Application No.: 09/506,942	)	Examiner: Shanon A. Foley
Filed: February 18, 2000	)	Confirmation No.: 9626
For: PHARMACEUTICAL	)	
COMPOSITION FOR TREATING	)	
PAPILLOMAVIRUS TUMORS AND	)	
INFECTION	)	

**DECLARATION BY JEAN-MARC BALLOUL UNDER 37 C.F.R. § 1.131**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

I, Jean-Marc Balloul, hereby state as follows:

1. I am a named inventor on U.S. Application No. 09/506,942 ("the '942 application").
2. I believe and allege the '942 application is a divisional application of U.S. Application Serial No. 09/043,933 ("the '933 application"), filed March 30, 1998, which claims priority benefit of French Application No. 96-09584, filed July 30, 1996.
3. I believe and allege we conceived the invention claimed in the '942 application prior to March 1996, and that conception was pursued with diligence from the time it was conceived before March 1996 until we filed the priority application, French Application No. 96-09584, on July 30, 1996.
4. Prior to March 1996, I participated in preparing an internal report for a scientific council at Transgene, attached as Exhibit A. This report discusses the production of clinical batches of vaccina virus construct VVTG5021 & 5065. This report further demonstrates expression of HPV genes and IL-2, the absence of

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toxicity seen in animal models following injection. The report also provides the same immunoprotein and immunotherapy assays as seen in Example 6 of the '942 application. The exact date of the internal report has been redacted on the copy provided. Further, data not connected to the '942 application has also been redacted on the copy provided.

5. Exhibit A, and the work detailed therein, was prepared in France, a World Trade Organization (WTO) member country, prior to March 1996.

6. We believe and allege each element of the claims of the '942 application is disclosed in Exhibit A.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date:

April 6<sup>th</sup>, 2006



JEAN-MARC BALLOUL

# EXHIBIT A

Scientific Council. TRANSGENE SA

Project N°87

Research and Development

Scientific Direction: MPK

Project Leader: JMB

Molecular Biology and Virology: MG, KD, ReB, JMB

Pre-clinical studies: NaB, CHP, LS, GiG, LR, BA

Production and controls: KD, EB, OF, EK, DV, HeL, JMB

## Cervical cancer associated with Human Papillomavirus

Among the 75 types of human papillomavirus types so far identified 20 distinct isolates are known to infect the genital tract and 4 types (HPV16, 18, 31 and 45) account for 80% of HPV infection associated with cervical cancer. Although cervical cancer rates have been declining, they remain relatively high in developing country where prevention is lacking. Both therapeutic vaccination (induction of the regression of precancerous and cancerous lesions associated with oncogenic HPV types), and prophylactic vaccination are under investigation in various laboratories throughout the world. Prophylaxis with HPV vaccines is certainly the most promising approach and will have a long term impact in reducing cervical cancer. At the moment a consensus exist for the choice of the immunogens to be include in prophylactic vaccines. They are in fact the virus-like particles (VLP) synthesised *in vitro*. These DNA free particles shown their ability to induce neutralizing antibodies. Several compaignies are now proceeding to the development and production of VLPs at the standards required for human trials.

### *Clinical batch for HPV16 invasive carcinoma immunotherapy*

As we predict that successful vaccination against the human papillomavirus requires induction of both cytotoxic T lymphocyte (CTL) response against early viral proteins and neutralizing antibodies against capsid proteins L1/L2 to prevent reinfection we developed a vaccinia virus expressing both early and late HPV antigens under the control of early late vaccinia virus promoters. Briefly HPV16 E7 and E6 have been mutated according the litterature in order to abrogate their transforming activity. Then mutant genes were inserted by homologuous recombination at the K1L locus of the vaccinia virus genome ( Copenhagen strain) and placed under the control of the early late vaccinia virus promoter H5R. At the same locus we inserted both the human Il2 gene placed under the control of the early late vaccinia virus promoter p7.5 and a selection marker gene LacZ placed under the control of the K1L promoter (see figure 1). Genes encoding for the late viral proteins L1 and L2 were inserted into the TK locus of the vaccinia virus genome and placed under the control of the vaccinia virus early-late promoter p7.5 (see figure 2).

Clinical batch was produced in our L3 unit on primary CEF. The viral amplification leads to the production of more than 3,000 doses of vaccines containing around 10E8 pfu per doses. Expression of HPV genes was analyzed by Western blot analysis. As shown in figure 3, 4,5 6, HPV antigens are expressed and detectable by Western blot. Level of Il2 was measured and evaluated to 10ng/ml/24h/10E6 cells infected with 1pfu per cell (see figure 7). Complete sequencing of open reading frames showed in the case of Il2 the accidental cloning of a 600bp

DNA fragment corresponding to the  $\beta$ -globin intron present in the parental plasmid. As 4 ORFs encoding for small polypeptides (26 to 56 aa) could be taken in account by the early-late vaccinia virus promoter controlling the expression of the human IL2 gene we analyzed the presence of such contaminants in VVTG5021&5065 infected BHK-21 extracts. Results showed that no contaminants are detectable compared with VVTG5021&5061 extracts expressing all genes present in the VVTG5021&5065 except the human IL2 gene (see figures 8, 9).

*New formulation of HPV16 early antigens in vaccinia virus*

### *MUC1/B7.1 formulation*

In order to enhanced immunogenicity of the MUC-1 antigen we constructed a new replicative vaccinia virus co-expressing the human B7.1 gene and the human MUC-1 cDNA (name code pTG6005 figure 13). MUC-1 cDNA available at Transgene contains more than 24 tandem repeats and regarding the propensity of the vaccinia virus genome to eliminate DNA repetitions we analyzed number of plaques after the first TK- selection. We isolate one clone containing at least 12 repeat units which is an impressive number compared to the VVTG5058 viral isolate.

## **Non Replicative Vaccinia Virus as Vector for Cancer Immunotherapy**

### *Introduction*

Vaccinia virus is the prototype of Orthopoxvirus. It can replicate in a wide range of cells. At least three viral genes affect the host range of orthopoxviruses in tissue culture: K1L gene, C7L gene and the cowpox 77-kDa gene. As regulatory authorities showed some fair concerning the use of replicative vectors for human health, we decided to develop attenuated poxvirus expression vectors. First question was to select the appropriate strain of vaccinia virus. Paolletti et al. has previously and extensively described various pox virus in their non replicative form such as NYCAC (highly attenuated vaccinia virus engineered from the Copenhagen strain) and ALVAC (Avian canaripox which is not replicative into mamalian cells). Unfortunately vectors for homologous recombination into these non replicative poxvirus were not accessible for Transgene. In the same time G. Sutter in B. Moss laboratory (Laboratory of viral diseases, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, USA) has described a recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus which has been shown to express the inserted foreign gene in authentic fashion and to provide the antigen to the immune system in such manner that it induces a protective immunity against pathogen such like the influenza virus in mice when it expresses haemagglutinin and nucleoprotein genes.

MVA known as modified vaccinia virus Ankara was found to be avirulent in normal or immunosuppresses animals ranging from rodents to macaques and was without significant side-effects in 120,000 humans, many of whom were at high risk for conventional smallpox vaccine. During over 570 passages in chicken embryo fibroblasts, MVA became host-restricted and unable to grow in almost all tested mamalian cell lines including those of rodent and human origin. Analysis of the genome revealed that viral DNA had suffered six major deletions resulting in the loss of 30000 base pairs equivalent of 15% of its genetic information. The replication of MVA DNA implied that the initial stages of infection comprising viral attachment, entry, early gene expression, and uncoating occurred in non permissive human cells. G. Sutter et al. has observed that only immature virus particles appearing as circular spiculecoated membranes encircling granular material and lacking dense nucleoprotein bodies could be seen by electron microscopic examination of human cells infected with MVA whereas mature brick-shaped particles with complex internal structures were numerous in human cells with WT or CEF with MVA. Moreover no increase in MVA titer was detected in Hela or 293 cell lines, and

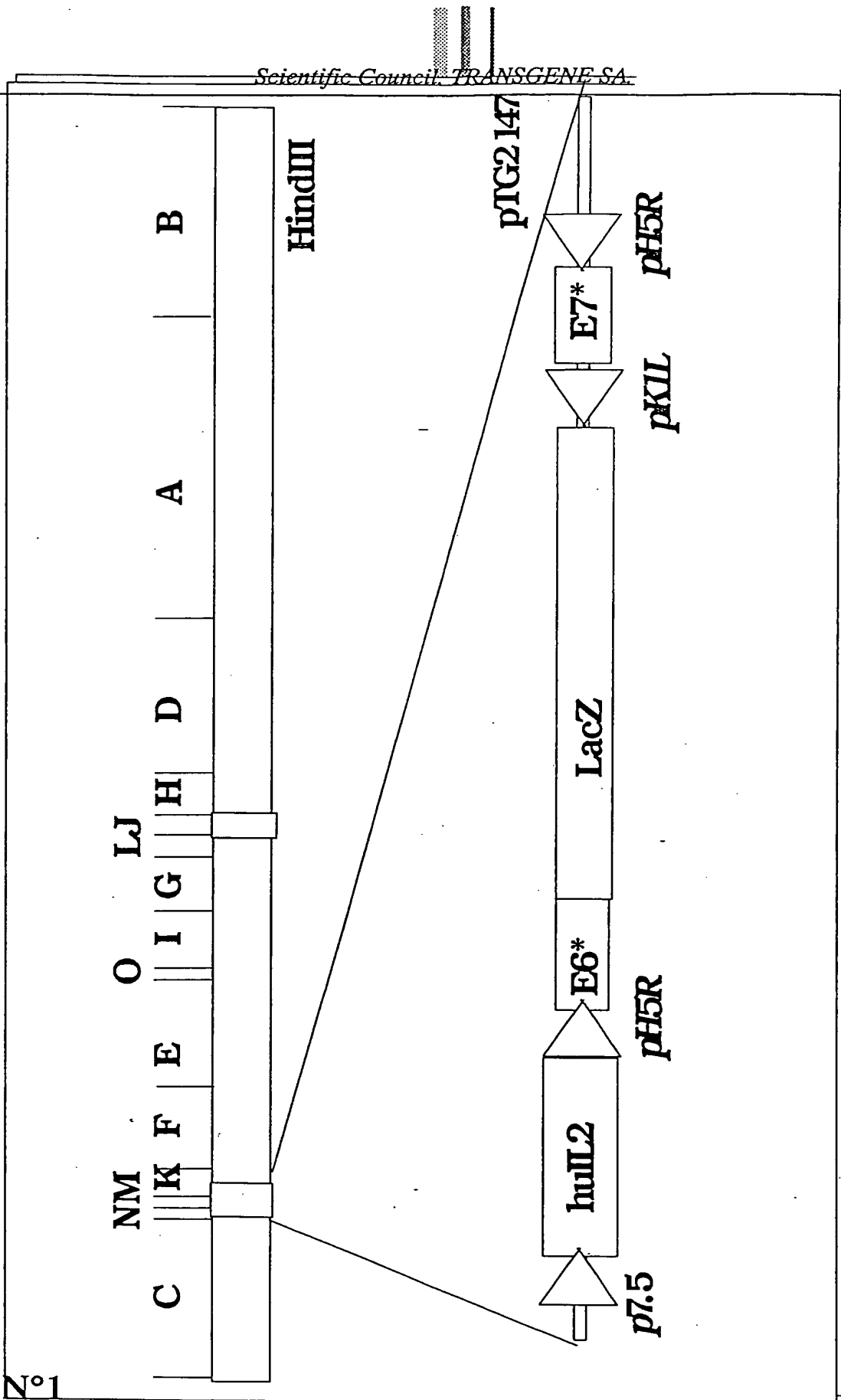
at the opposite WT increase 10,000-fold in titer in the same conditions. These differences are not associated with DNA replication because concatemeric forms of replicative MVA DNA are processes normally to unit genome. These authors observed common viral protein pattern for both WT and MVA in infected human cells with one exception for a 90kDa polypeptide which is not seen in the MVA protein pattern. This is attributed to the deletion of the gene encoding nonessential A-type inclusion protein homolog. In pulse-chase assays they also showed that if in WT infected Hela cells expected cleavage of at least 5 polypeptides including the major core protein precursors P4a and P4b occurred, the processing of MVA proteins was inhibited.

#### *Plasmid construction*

PCR segments flanking either deletion III or deletion II were cloned into pTG1E at the unique EcoRI site (code name pTG6018 containing flanking DNA segments for homologous recombination at the deletion II site and pTG6019 containing flanking DNA segments for homologous recombination at the deletion III site figure 14). In order to facilitate the isolation of recombinant viruses we inserted between these flanks a double expression cassette composed of the early late promoter p7.5 controlling the marker gene and of the early late promoter p7.5 in the same orientation controlling a reporter gene for internal recombination which encode for the human Il2. We have selected as markers either the LacZ gene encoding  $\beta$ -galactosidase ( $\beta$ -gal) in the case of deletion II and the E. coli *gusA* gene encoding  $\beta$ -glucuronidase (GUS) in the case of the deletion III.  $\beta$ -gal and GUS being from E. coli origin we decided to eliminate these genes from the final clinical product. For these purpose we used the natural propensity of the vaccinia virus to eliminate DNA repeated sequences. Therefore p7.5 flanking marker gene should recombine properly, excising the E. Coli gene and reconstituting a functional p7.5 promoter controlling the human Il-2 gene (figure 15).

# VVTG5065

Figure N°1



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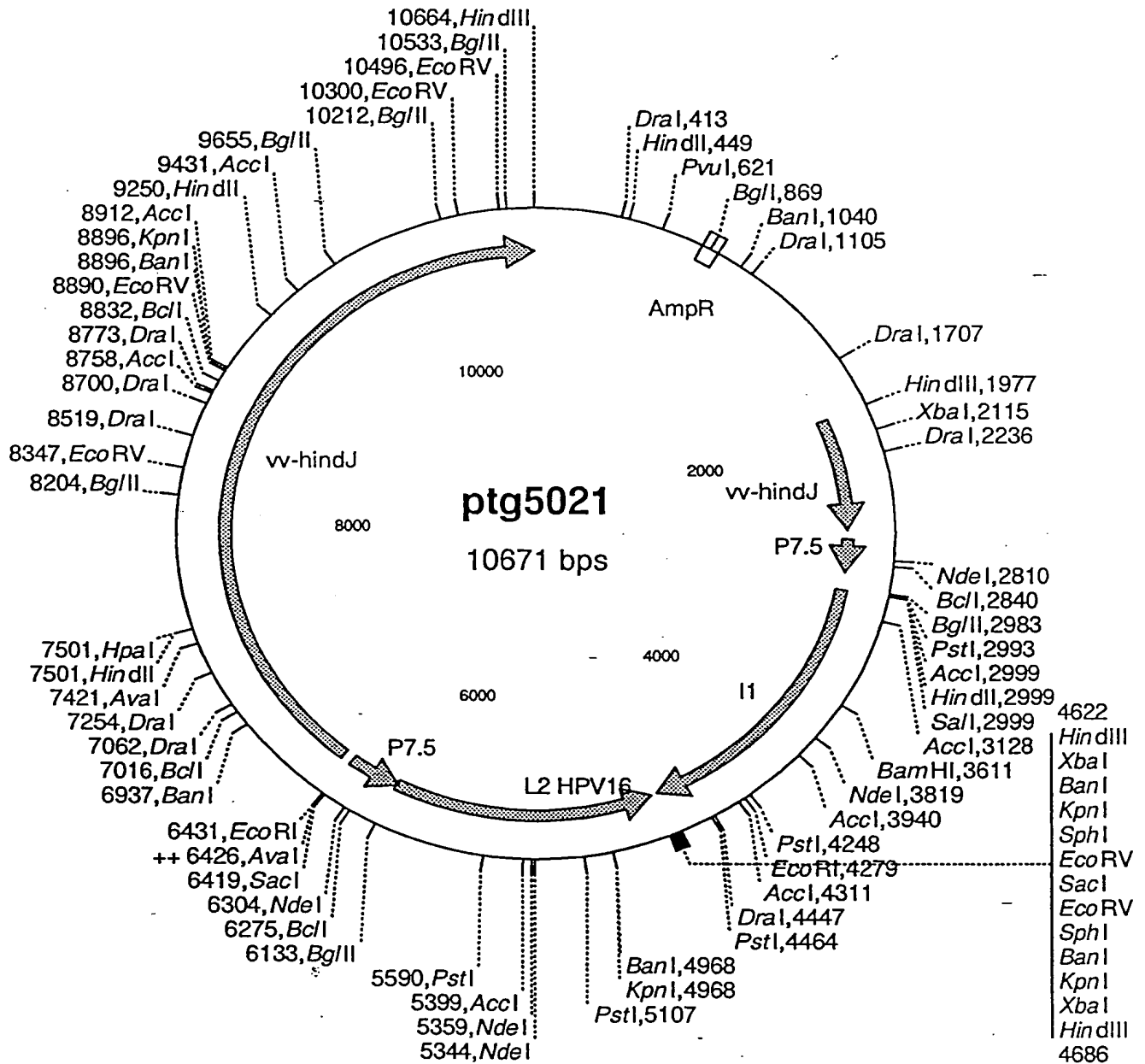
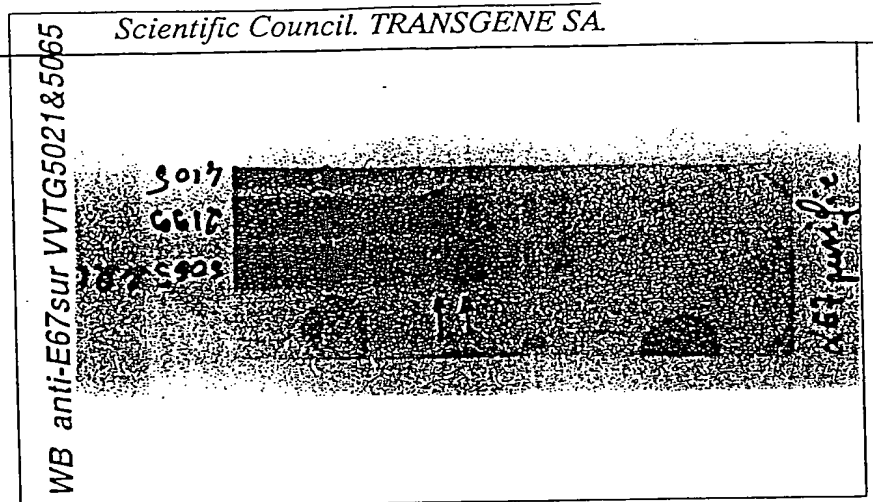


Figure N°2





Figure N°4



**Séquence pTG5021&5065**

Similarity index = 99.637, total bases not opposite a gap = 279  
 Number of gaps = 1, bases opposite gaps = 18  
 Length of the overlapping region = 277  
 Number of unambiguous matching bases = 279  
 Number of ambiguous matching bases = 0

L1 produite par  
VVTG5021&5065

A schematic diagram of a two-layer system. The top layer is labeled L2 and the bottom layer is labeled L1. The interface between them is marked with p7.5 on both sides. Arrows indicate flow direction from left to right.

WB / CAM-1 sur VVTG5021&amp;5065

5905  
WTG5021

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L2 produite par  
VVTG5021&5065

7.5 11 L2 7.5

WB anti-L2 lapin sur VVTG5021&amp;5065

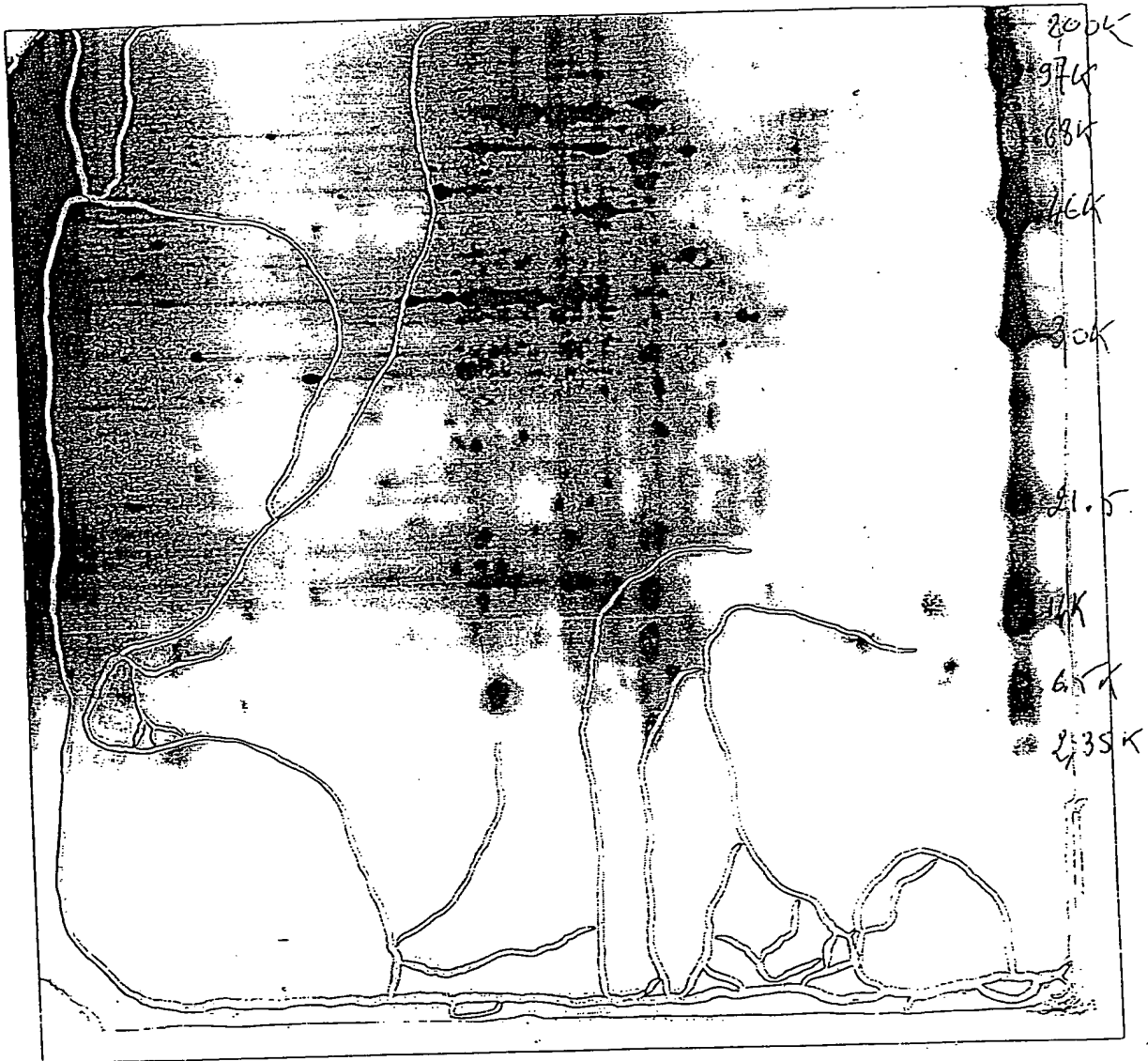
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WB anti-L2 lapin sur VVTG5021&amp;5065

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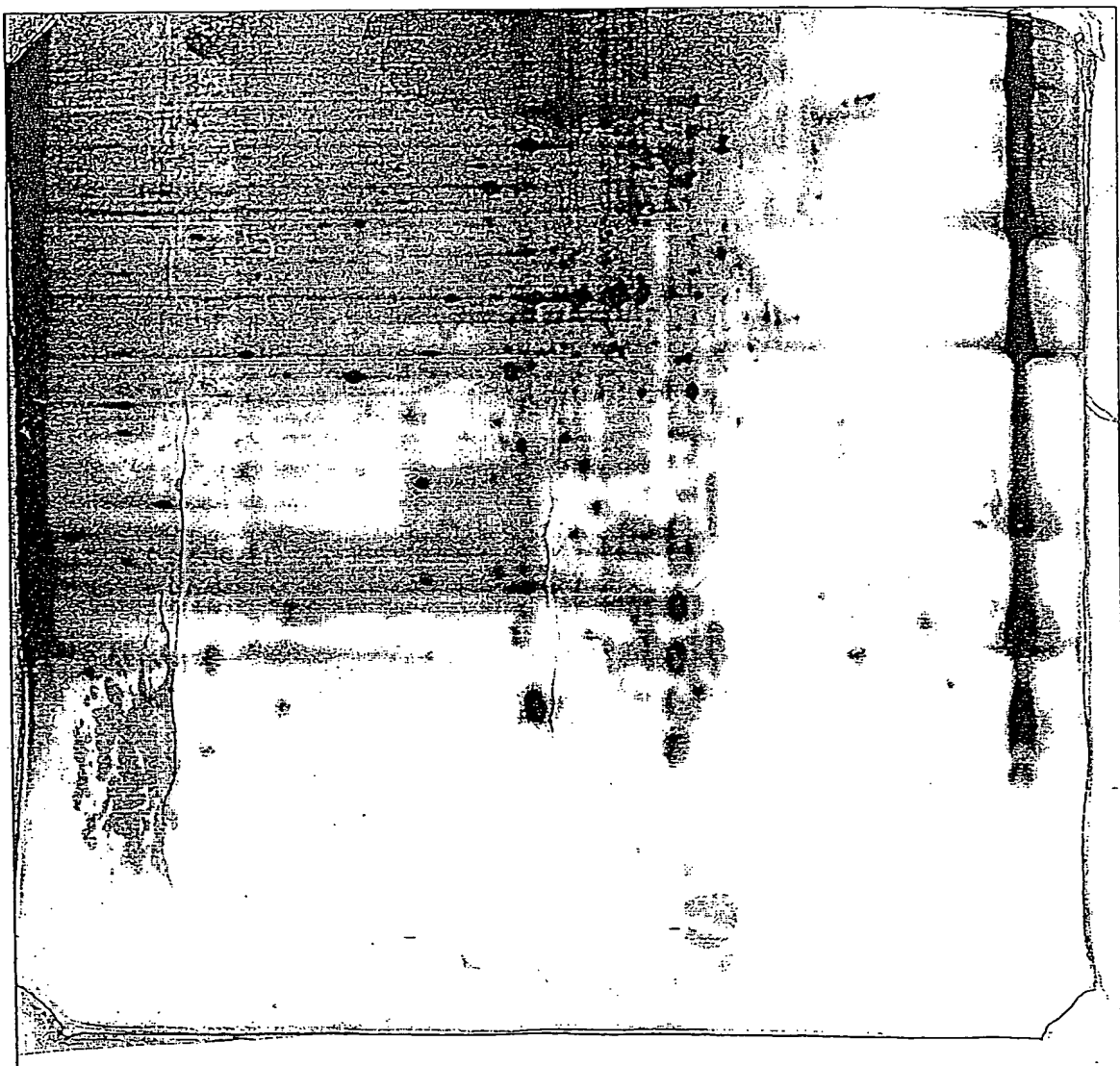




Nitrate d'argent, fixation des  
petites protéines par la  
formaldéhyde

# VVTG5021&5061

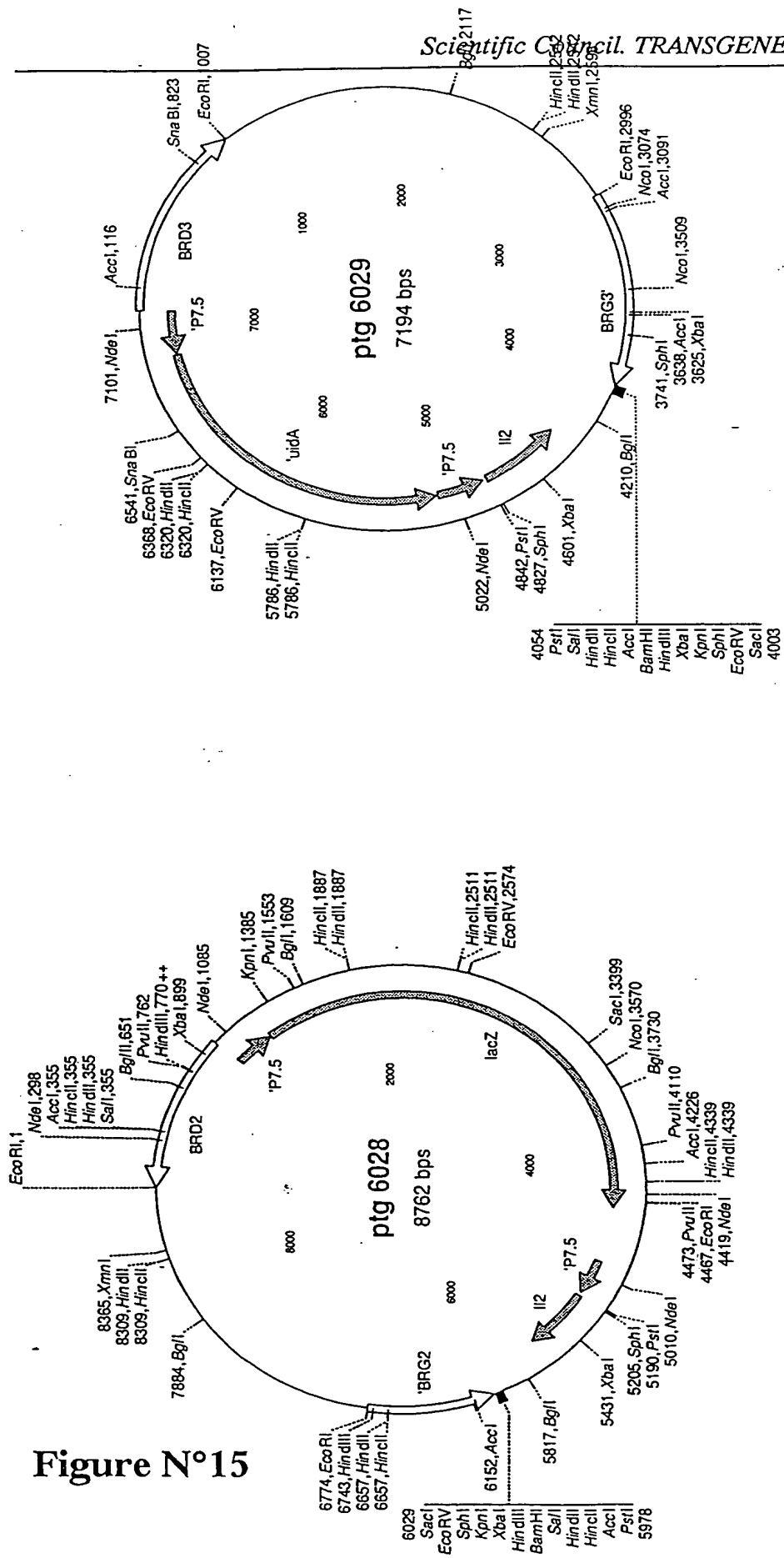
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Nitrate d'argent, fixation des  
petits peptides par la  
formaldéhyde

Figure N°9

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**Figure N°15**



Nadine Bizouarne, Catherine Pêcheur

Scientific council,

## Project 87 : Animal models for vaccinia based vectors for specific cancer immunotherapy.

Infection of mucosal epithelium by papillomaviruses plays a critical role in the development of genital and oral warts and is implicated in the induction of human oropharyngeal and cervical cancer. There are actually no vaccine to prevent disease caused by HPV infection. Different levels of problems are encountered in the development of vaccine strategies. First, these viruses are highly species-specific and their *in vitro* culture is, up to now, impossible, making the use of a "simple" animal model out of the question. All the experiments described in this report were done in an "artificial" animal model consisting of mice grafted with tumor cells expressing an HPV target molecule (E7). Second, the HPV uses several strategies to escape its host immune system. In particular i) mucosal lesions caused by HPV induce small quantities of viral particle (the induction of an humoral immune response seems to be only complementary to a cellular one), ii) during the transformation event of the host cell, the viral DNA is integrated to the cellular genome and only the viral early proteins continue to be expressed at low levels, reducing our choice for antigen-specific immunotherapy. As described previously by JM Balloul, a recombinant vaccinia virus expressing E6\*E7\*L1L2.L2 (non oncogenic mutated forms of E6 and E7 proteins, VV5021X5065) was produced. In this report we will describe the *in vivo* activities of this vaccinia vector and several approaches used to obtain a second generation vaccinia virus vector.

### 1- *In vivo* toxicity of VV5021X5065 :

In order to demonstrate the attenuation of the VV5021X5065, we compared the toxicity of this construct to the toxicity of a wild type vaccinia virus. This comparison was made in immunodeficient mice.

**1.1 Dose effect :** Nude mice were injected intramuscularly with  $10^6$ ,  $10^7$  or  $10^8$  pfu of VV5021X5065 or VVwt. The number of mice developing vaccinia lesions are indicated in the table 1, and show that even when injected with the non attenuated VVwt, few mice have vaccinia lesions.

**1.2 Immunization route effect :** Nude mice were injected with  $10^7$  pfu of vaccinia by different routes of immunization : intracranial , intramuscular, intravenous, or subcutaneous. The table 2 represents the number of mice having vaccinia lesions:

It seems clear that the VV5021X5065 is consistently attenuated comparing to the VVwt: even when injected by the intracranial route, this recombinant does not lead to vaccinia lesions in this experimental system.

VV	WT	IN S/S	VV5021X5065	0/5
		IC		0/5
		IP		0/5
		TH		0/5

... VV recombinant attenuated

## 2- *In vivo* antiviral effect of Ribavirine on vaccinia virus :

In order to make a clinical trial with a recombinant vaccinia virus, we have to test the ability of an antiviral compound to inhibit the development of vaccinia lesions. As we have previously seen, when mice are injected with VV5021X5065, they do not develop vaccinia lesions (even immunodeficient mice). Therefore, the antiviral efficiency was tested in nude mice injected with VVwt and treated with Ribavirine. The table 3 shows the number of mice with lesions after antiviral treatment, and shows that an *in vivo* efficient anti-vaccinia compound is available.

## 3- *In vivo* efficacy of VV5021X5065:

The viral construct VV5021X5065 was tested for *in vivo* anti-tumoral activity against cells expressing the E7 molecule, and was compared to VV5021X5061 (E6\*E7\*L1L2), VVIL2 and VVcontrol (186). This activity was assessed in 2 models : immunoprotection and immunotherapeutic experiments, whose protocols are similar in all experiments described in this report.

**3.1 - Immunoprotection experiment :** Mice were immunized subcutaneously 3 times with  $10^7$  pfu of VV5021X5065. Three days after the last immunization, they were challenged with  $10^3$  E7W1 cells. The percentage of surviving animals in function of time is represented in figure 1 and shows a clear increase for mice immunized with VV5021X5065.

	$10^6$ pfu	$10^7$ pfu	$10^8$ pfu
VVwt	0/5*	1/5	1/5
VV5021X5065	0/5	0/5	0/5

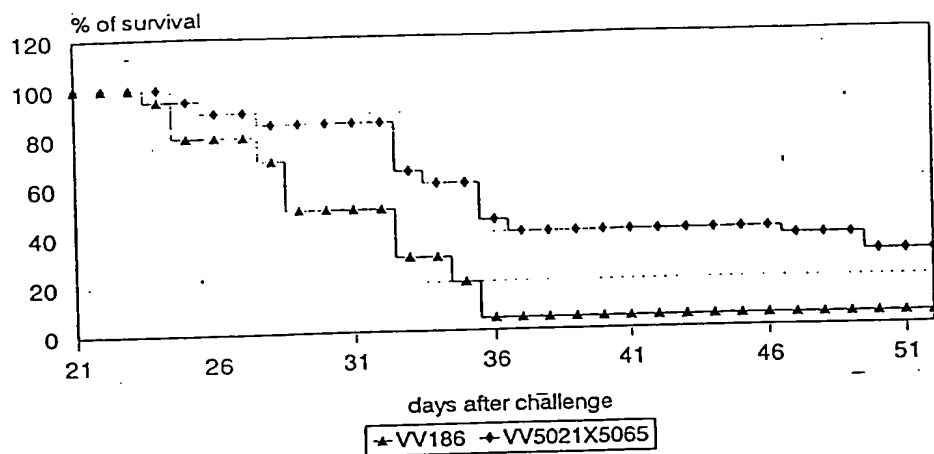
Table 1 : Toxicity of VV5021X5065 compared to VVwt - Dose effect. \*number of mice with lesions .

	IV	IP	IM	IC
VVwt	5/5 (2 dead D16)	3/5	1/5	4/5 (4 dead D5)
VV5021X5065	0/5	0/5	0/5	0/5

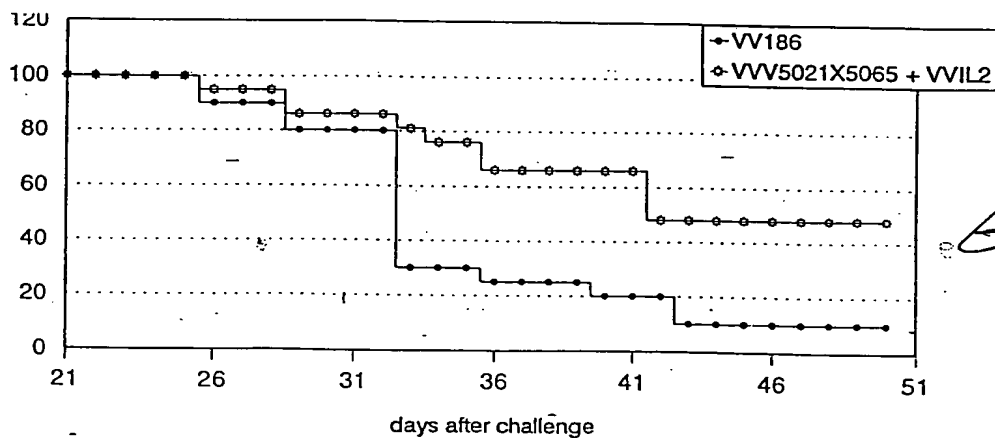
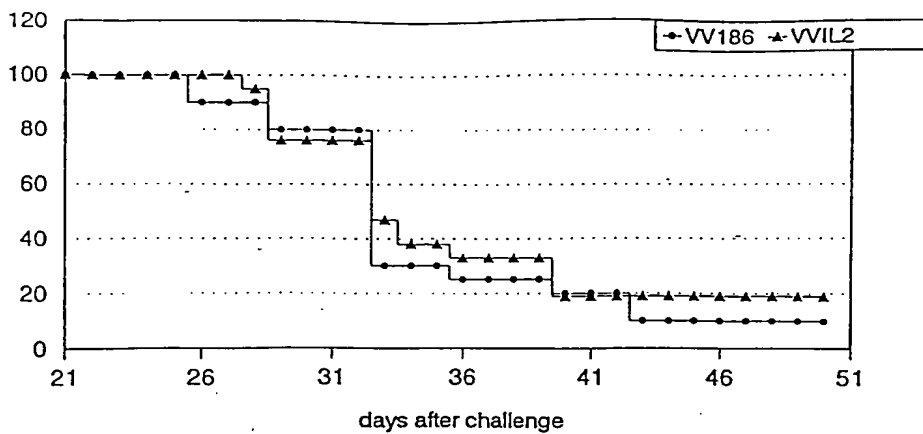
Table 2 : Toxicity of VV5021X5065 compared to VVwt - Immunization route effect. \*number of mice with lesions.

	control		Ribavirine (30mg/kg)		Ribavirine (300mg/kg)	
n° mice	fingers	tail	fingers	tail	fingers	tail
1	0	0	++++	+	0	0
2	0	+	0	+	0	0
3	+++	+++	+++	+	0	0
4	+++	+++	0	0	0	0
5	0	+	+	+	0	0
6	++	+++	+++	++	0	0
7	0	+	++++	++	0	0
8	0	0	+++	++	0	0
9	+++	+++	+	0	0	0
10	+++	+++	0	0	0	0

Table 3 : *In vivo* antiviral effect of Ribavirine on wild type vaccinia virus lesion in nude mice.



**Figure 1 :** In vivo efficacy of VV5021X5065. Immunoprotection experiment.  $10^7$  pfu VV SC,  $3 \times 10^3$  E7W1 SC.



← + combinaison de virus

Figure 7 : Immunotherapy experiment. Effect of IL2 dosage. Mice were injected with  $10^3$  E7W1 cells and then treated 3 times with  $10^7$  pfu of VV (SC).

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